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# Dendritic cell vaccination as post-remission treatment to prevent or delay relapse in acute myeloid leukemia

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## KEY POINTS

- *WT1* mRNA-electroporated dendritic cells can prevent or delay relapse in 43% of AML patients in remission after chemotherapy.
- OS compares favorably with the new survival data from the SALR, and correlates with molecular and *WT1*-specific CD8<sup>+</sup> T-cell responses.

## ABSTRACT

Relapse is a major problem in acute myeloid leukemia (AML) and adversely impacts survival. In this phase II study, we investigated the effect of vaccination with dendritic cells (DCs) electroporated with *Wilms' tumor 1* (*WT1*) mRNA as post-remission treatment in 30 AML patients at very high risk of relapse. There was a demonstrable anti-leukemic response in 13 patients. Nine patients achieved molecular remission as demonstrated by normalization of *WT1* transcript levels, 5 of which are sustained after a median follow-up of 109.4 months. Disease stabilization was achieved in 4 other patients. Five-year overall survival (OS) was higher in responders than in non-responders (53.8% vs. 25.0%;  $P=0.01$ ). In patients receiving DCs in first complete remission (CR1), there was a vaccine-induced relapse reduction rate of 25% and the 5-year relapse-free survival was higher in responders than in non-responders (50% vs. 7.7%;  $P<0.0001$ ). In patients  $\leq 65$  and  $>65$  years who received DCs in CR1, 5-year OS was 69.2% and 30.8% respectively, as compared to 51.7% and 18% in the Swedish Acute Leukemia Registry (SALR). Long-term clinical response was correlated with increased circulating frequencies of poly-epitope WT1-specific CD8<sup>+</sup> T-cells. Long-term OS was correlated with interferon- $\gamma$ <sup>+</sup> and tumor necrosis factor- $\alpha$ <sup>+</sup> WT1-specific responses in delayed type hypersensitivity-infiltrating CD8<sup>+</sup> T-lymphocytes. In conclusion, vaccination of AML patients with *WT1* mRNA-electroporated DCs can be an effective strategy to prevent or delay relapse after standard chemotherapy, translating into improved OS rates, which are correlated with the induction of WT1-specific CD8<sup>+</sup> T-cell response. This trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as #NCT00965224.

## INTRODUCTION

Acute myeloid leukemia (AML) still has a dismal prognosis.<sup>1,2</sup> According to the latest data of the Surveillance, Epidemiology and End Results (SEER) program of the National Cancer Institute,<sup>1</sup> the 5-year overall survival (OS) of AML is only around 25%. One of the major reasons for this is that the majority of patients relapse even after complete remission (CR) is achieved with standard chemotherapy.<sup>3</sup> Relapse is usually caused by the persistence of a small population of residual leukemic cells, a condition designated as minimal residual disease (MRD).<sup>4</sup> Allogeneic hematopoietic stem cell transplantation (allo-HSCT), the best established post-remission treatment to eradicate MRD, decrease the risk of relapse and increase survival following chemotherapy, is still beset by substantial morbidity and mortality.<sup>5</sup> As a consequence, allo-HSCT is generally not considered as a therapeutic option in the large group of older AML patients. For these patients and for younger patients without a compatible donor, there is currently no standard adjuvant treatment to prevent post-chemotherapy relapse.<sup>4</sup>

The beneficial effect of allo-HSCT against leukemia is mediated in large part by T-cells that are capable of recognizing antigens expressed on the leukemia cells and of subsequently mediating AML cell killing.<sup>6</sup> Stimulation of autologous T-cells by *in vivo* immunization with leukemia-associated antigens is an innovative strategy to combat relapse in AML,<sup>7-11</sup> acting via the reduction or eradication of MRD. Several antigens have been identified to serve as T-cell targets in AML, including the Wilms' tumor protein 1 (WT1) which is highly overexpressed in AML and is also involved in leukemogenesis.<sup>12</sup> In view of their role as the most potent antigen-presenting cells of the immune system, dendritic cells (DCs) are eminently equipped to stimulate antigen-specific T-cell immunity.<sup>13</sup> This explains the strong interest in the use of these cells for cancer vaccination strategies.<sup>14</sup>

The aim of this phase II study was to determine the clinical efficacy of DC vaccine therapy in AML, and, more specifically, whether this form of immunotherapy can be applied in the adjuvant setting to decrease the risk of relapse following chemotherapy and to improve survival. To this end, we here vaccinated 30 AML patients in remission but at very high risk of relapse with autologous DCs loaded with the WT1 antigen by means of mRNA electroporation, a technique that allows for human leukocyte antigen (HLA) haplotype-independent, multi-epitope antigen presentation to T-cells.<sup>15,16</sup>

## METHODS

### Patients

Thirty AML patients were enrolled in this phase II study (ClinicalTrials.gov identifier NCT00965224), whereby the first 10 patients were also included in a preceding feasibility, safety and immunogenicity study (ClinicalTrials.gov identifier NCT00834002).<sup>17</sup> This study was approved by the ethics committee of the Antwerp University Hospital (UZA), Edegem, Antwerp, Belgium. Enrollment criteria for the phase II study were adult patients with AML (except acute promyelocytic leukemia), diagnosed according to World Health Organization (WHO) criteria, in remission after at least one course of polychemotherapy and at high risk of relapse as defined by: (i) age >60 years (yr) or if younger than 60 yr without matched sibling donor for allo-HSCT; (ii) poor risk cytogenetic or molecular markers; (iii) hyperleukocytosis at presentation; and/or (iv) previous relapse.

### DC vaccination

Clinical grade *WT1* mRNA-electroporated DC (WT1/DC) vaccines were prepared and administered intradermally as described previously.<sup>16,17</sup> Three different *WT1* constructs were used to generate mRNA by *in vitro* transcription (Figure 1, Table S1): construct 1 ("WT1"), encoding full-length *WT1*;<sup>16,17</sup> construct 2 ("WT1-DC-LAMP"), incorporating a Sig-DC-LAMP major histocompatibility complex (MHC) class II-skewing signal with deletion of the *WT1* nuclear localization signal (NLS); and construct 3 ("WT1-DC-LAMP-OPT"), a codon-optimized version of construct 2.<sup>18</sup>

### Molecular tumor marker monitoring and clinical response criteria

Longitudinal monitoring of *WT1* transcripts was performed as described previously using an in-house assay,<sup>17,19</sup> the *ipsogen* WT1 ProfileQuant Kit (Qiagen)<sup>20</sup> or the WT1 mRNA OneStep Assay (Otsuka Pharmaceutical Co).<sup>19</sup> *WT1* mRNA levels above background (respectively above 1 and 25 copies of *WT1* mRNA per 1,000 *ABL* copies in blood and marrow in the in-house assay; according to manufacturer's instructions in the commercial kits) are indicative of MRD and herald full relapse.<sup>21-25</sup> Responders were characterized by molecular remission (MR) or by stable disease (SD). MR was defined by normalization of *WT1* mRNA levels in blood and/or bone marrow during WT1/DC vaccination, while reaching or maintaining hematological CR. SD was defined by stable *WT1* blood transcript levels above background, with stable blood values without blasts. The minimum duration of SD was 2 months, after which *WT1* mRNA levels did not increase by more than a factor of 0.5 log<sub>10</sub> and at least 1 basic blood value (hemoglobin, thrombocyte count and/or absolute neutrophil count) was normal. Patients who relapsed without achieving MR or SD status were

categorized as non-responders. The classical definitions of CR, partial remission (PR), relapse and survival were used.<sup>26</sup> Long-term survivors were defined as surviving for at least 3 yr<sup>26,27</sup> and long-term responders as patients with a MR or SD response remaining in CR for at least 3 yr, after the first dose of WT1/DCs.

### Immunomonitoring

Detection and subtyping of anti-WT1 antibodies in pre- and post-vaccination plasma samples was performed as described previously.<sup>17,28</sup> The increase in WT1 IgG antibody levels after vaccination was determined by subtraction of the corresponding pre-vaccination values.

Cytokine plasma levels were determined using the Th1/Th2 multiplex immunoassay (Bender MedSystems). *Ex vivo* flow cytometric analysis of lymphocyte subsets was performed using directly conjugated monoclonal antibodies (BD Biosciences).

Circulating WT1-specific CD8<sup>+</sup> T-cells obtained before vaccination and after the fourth dose of WT1/DCs were stained with peptide-HLA-A\*0201 tetramers and quantified as described previously.<sup>17,29</sup> Whenever cells were available they were also analyzed at different time points afterwards until relapse and/or progression.

Two weeks after the 4th WT1/DC vaccination, delayed-type hypersensitivity (DTH) was tested against the complete vaccine (i.e. keyhole limpet hemocyanin [KLH]-exposed WT1/DCs, except in patients UPN11, 12, 13 where non-KLH-exposed WT1/DCs were used). This was performed by intradermal injection of  $0.5 \times 10^6$  WT1/DCs on the back of the patient. Forty-eight hours later, erythema and induration at the injection site were measured and skin punch biopsies were taken for culture of DTH-infiltrating lymphocytes (DILs). These DILs were allowed to expand for 2-3 weeks in medium with interleukin (IL)-2 (100 IU/mL). Expanded DILs were then harvested and tested for antigen specificity as described previously.<sup>17</sup>

### Data mining and statistical analysis

Statistical calculations and data graphing were carried out using Prism version 5.01 (GraphPad). A *P* value <0.05 was considered statistically significant.



## RESULTS

### Patients

WT1/DC vaccination as a post-remission treatment was evaluated in 30 high-risk AML patients in remission following chemotherapy. Patient characteristics with baseline evaluation and initial treatments are shown in Table S1. There were 15 males and 15 females, with a median age at diagnosis of 65 yr. Prior to WT1/DC vaccination, 27 patients had achieved CR following chemotherapy, whereas 3 had partial remission (PR). Six patients had a preceding hematological disorder, myelodysplastic syndrome (n=5) or myeloproliferative neoplasia (n=1). The cytogenetic risk group<sup>30</sup> was adverse in 1, intermediate in 23 and favorable in 6 patients. All patients were at increased risk of relapse, as indicated by the unfavorable prognostic features in Table S1. In particular, *WT1* transcript levels above background post-induction and/or post-consolidation chemotherapy were predictive of relapse<sup>21-25</sup> in 5/6, 18/23 and 1/1 of patients with respectively favorable, intermediate and adverse cytogenetic risk. Of the remaining 6 patients without increased *WT1* transcript levels after chemotherapy, only 1 with erythroleukemia in the intermediate risk group (UPN22) did not relapse and his response was categorized as undefinable (Figure 1).

### Clinical response

There was a demonstrable anti-leukemic effect of the WT1/DC vaccination only without any concomitant chemotherapy in 13/30 patients, corresponding to a clinical response rate of 43%. Of these 13 patients, 9 went into molecular remission (MR) as demonstrated by the normalization of *WT1* transcript levels in blood and/or bone marrow (UPN01, 06, 08, 10, 11, 14, 15, 16, 17). Of these 9 patients, 2 went from PR to CR (UPN08, 16),<sup>17</sup> and 5 are still in CR (UPN06, 08, 10, 14, 15) with a median duration of 114.5 months (mo) and a median follow-up after the first WT1/DC vaccination of 109.4 mo. Four patients relapsed after reaching MR, 3 ultimately dying of AML (UPN01, 11, 16) and 1 achieving long-term CR after undergoing allo-HSCT (UPN17). In all relapsing patients, recurrence of AML was preceded and accompanied by increased *WT1* transcript levels. In the remaining 4 patients (UPN21, 33, 35, 48), the clinical response was characterized by stable disease (SD) as demonstrated by elevated but stable *WT1* transcript levels in blood and stable blood values without blasts. An example of SD is shown in Figure 2. The stable *WT1* profile seen during SD contrasts with the steeply rising curve that is usually seen when AML patients are relapsing.<sup>19,23,25</sup>

Only a minority of patients had a molecular MRD marker other than *WT1* mRNA. Overall, there was a corresponding evolution, between the *WT1* transcript levels and other markers of MRD, such as the fusion transcripts *RUNX1-RUNX1T1* (translocation t(8;21)) and *CBFB-MYH11* (inversion inv(16)). For instance, in clinical responder UPN15, the

normalization of bone marrow *WT1* expression after 4 WT1/DC vaccinations (from 638 to 4 copies/1000 *ABL* copies) was paralleled by a decrease of *RUNX1-RUNX1T1* transcript levels from 1.43 to 0.0495 copies/1000 *ABL* copies. In non-responder UPN28, the increase in already elevated blood *WT1* transcript levels (from 24.1 to 1195 copies/1000 *ABL* copies) was mirrored by an increase of *CBFB-MYH11* levels from 10 to 469 copies/1000 *ABL* copies.

The clinical response rate was 50% in patients with favorable cytogenetic risk (MR in 3/6 patients). Among the 22 treatment response-evaluable patients with intermediate cytogenetic risk, 6 patients experienced a MR and 4 other patients SD, corresponding to a clinical response rate of 45%. The response to WT1/DC vaccination, grouped according to the *WT1* construct used, is indicated in Figure 1 and Table S1. Of the 29 patients with a definable response or non-response, there was an effect in 8/16 in the “WT1” group, 3/5 in the “WT1-DC-LAMP” group and 2/8 in the “WT1-DC-LAMP-OPT” group. Of the 3 patients receiving DCs prepared without KLH (UPN11, 12, 13), 1 achieved MR (UPN11).

Overall, 6/30 patients (UPN06, 08, 10, 14, 15 and 22) have not relapsed yet and are still in CR1 with a median duration of 107.6 mo and a median follow-up of 101.8 mo after the first dose of WT1/DCs. Of the remaining 24/30 patients, 1 did not reach CR1 (UPN20), 1 died presumably of a lung adenocarcinoma without morphological evidence of AML relapse (UPN07), 3 had a second or third relapse (UPN02, 16, 34) and 19 a first relapse. Four of these 19 AML patients in first relapse received supportive care (UPN01, 11, 21, 38), whereas the remaining 15 patients were treated with salvage therapy (chemotherapy with or without allo-HSCT; Table S1). The CR2 rate in this group was 73.3% (11/15 patients: UPN05, 12, 13, 17, 28, 30, 35, 36, 46, 47, 48); the remaining 4 patients had progressive disease (UPN03, 09, 29, 33).

## Survival

Survival data are shown in Figures 1 and 3, and Tables 1 and S1. The 5-yr relative OS data compared favorably with those reported by the SEER program of the National Cancer Institute.<sup>1</sup> OS and relapse-free survival (RFS) were significantly higher in responders as compared to non-responders, irrespective of age categories. The OS data were not influenced significantly by allo-HSCT, which was carried out in some patients who relapsed during WT1/DC vaccination (data not shown).

Of the patients in first CR (CR1; Table 1) who received WT1/DCs, survival from diagnosis compared favorably with that of patients from the Swedish Acute Leukemia Registry (SALR), a comprehensive population-based AML database.<sup>31</sup> The 5-yr relative RFS of AML patients in CR1 treated with WT1/DCs was similar to that of the SALR. It should be noted that the risk of relapse in the patients treated with WT1/DCs was most probably higher than that of SALR patients, part of whom (overall 32.1%) did not relapse after 5 yr (Table 1).

In contrast, 25/26 patients who were vaccinated with WT1/DCs were destined to relapse based on increased pre-vaccination *WT1* transcript levels and/or relapsed. The RFS of responding patients was markedly better than the RFS of patients from the SALR. Of the 25 response-evaluable patients in CR1, 5 have not relapsed yet (UPN06, 08, 10, 14, 15; all of them responders), compatible with a vaccine-induced relapse reduction rate of 25%. The median duration of CR1 in these 5 patients has been indicated above. There were 16/30 patients in the long-term survivor category (UPN01, 06, 08, 10, 11, 12, 14, 15, 17, 21, 22, 28, 29, 33, 34, 35).

Long-term survival was observed in 11/13 responders and in 4/16 non-responders; the difference is significant ( $P=0.0025$ , Fisher's exact test), correlating long-term survival with response to WT1/DCs. Long-term survival was noted in 5/6 and 10/22 response-evaluable patients with respectively favorable and intermediate cytogenetic risk; the difference between the 2 groups was not significant ( $P=0.20$ ). There were no significant differences in OS outcome between the different *WT1* constructs used (Figure 1; "WT1" vs. "WT1-DC-LAMP",  $P=0.48$ ; "WT1" vs. "WT1-DC-LAMP-OPT",  $P=0.44$ ; "WT1-DC-LAMP" vs. "WT1-DC-LAMP-OPT",  $P=0.84$ ; "WT1" vs. "WT1-DC-LAMP" + "WT1-DC-LAMP-OPT",  $P=0.34$ ).

As shown in Table S1, as of December 31, 2016, 11/30 patients were alive in CR with a median OS from diagnosis of 99.4 mo (range 72.6 – 125.5 mo). Of these 11 patients, 6 were alive in continuing CR1 (UPN06, 08, 10, 14, 15, 22). Five other surviving patients who relapsed after WT1/DC vaccination were brought back into CR by chemotherapy followed (UPN17, 28) or not (UPN12, 34, 35) by allo-HSCT. Remarkably, patients UPN12 and 34 are alive in continuing CR respectively more than 7 yr and 4 yr, after achieving CR2 and CR3 with chemotherapy alone.

Of the 19 AML patients, who were in first relapse after WT1/DC vaccination, 52.6% were alive at 3 yr and 36.8% at 5 yr from diagnosis. Of the latter patients, 4 achieved CR2 following chemotherapy (UPN12, 17, 28, 35), while the other 3 had a remarkably long period of CR1 before relapsing (UPN01, 11, 29: respectively 47, 51.6 and 59.8 mo after the start of WT1/DC vaccination).

### **WT1/DC vaccine-induced immune responses**

Immunomonitoring was performed on PBMCs, DILs and plasma samples obtained before and/or after WT1/DC vaccination. There were no significant changes after vaccination with respect to: (i) the frequencies or absolute numbers of circulating lymphocyte subsets ( $CD4^+$  and  $CD8^+$  T-cells, B-cells and natural killer cells); (ii) the relative frequencies of naïve, terminally differentiated effector, effector memory, central memory subsets within the  $CD4^+$  and  $CD8^+$  T-cell compartments; (iii) the relative frequencies of regulatory T-cell subsets; or

(iv) the relative frequencies of myeloid-derived suppressor cells. T-helper (Th)1/Th2 cytokine levels and anti-WT1 IgG titers in plasma were also unchanged after WT1/DC vaccination.

WT1-specific T-cell responses were evaluated using pHLA-A\*0201 tetramer staining and intracellular cytokine assays. Increased ( $>1.5$ -fold) frequencies of WT1-specific tetramer<sup>+</sup> CD8<sup>+</sup> T-cells were observed after vaccination in 6/12 evaluable (HLA-A\*0201<sup>+</sup>) patients. A significant positive correlation ( $P=0.018$ ) was found between long-term clinical response (UPN01, 08, 35) and increased circulating frequencies of poly-epitope WT1-specific tetramer<sup>+</sup> CD8<sup>+</sup> T-cells (Table S2). Of the 9 HLA-A\*0201<sup>+</sup> non-responder patients, only UPN30 had increased numbers of poly-epitope WT1-specific CD8<sup>+</sup> T-cells. The other non-long-term responders showed either no (6/9 patients) or mono-epitope (2/9 patients) WT1-specific CD8<sup>+</sup> T-cell responses to WT1/DC vaccination. WT1-specific tetramer<sup>+</sup> CD8<sup>+</sup> T-cells were also assessed in 7 HLA-A\*0201<sup>+</sup> patients (UPN16, 17, 30, 34, 35, 38, 47) at relapse or disease progression: the frequencies were not lost in any patient upon relapse. In all but one patient (UPN16), we observed an increase of frequencies for at least 2 epitopes as compared to the post 4<sup>th</sup> WT1/DC vaccination sample and in all patients a maintenance and/or increase for the other epitope(s) examined (data not shown).

Because KLH was shown in our previous study<sup>17</sup> to skew the T-cells towards a Th2 profile, which could be detrimental for a cytotoxic antitumoral response, it was omitted from the preparation of the DCs in 3 patients (UPN11, 12, 13). In those patients, there was no local immunoreactivity at the site of DC injection, nor was there any DTH reactivity and it was thus impossible to assess *in vivo* the quality of the DCs for their capacity to migrate to the lymph nodes and to elicit T-cell response. For this reason, it was therefore decided to reincorporate KLH in the DC vaccine preparation in all other patients. All the KLH-exposed patients showed a DTH response; in 13 of them, DILs were obtained for immune response assessment. Functional analysis of DILs restimulated with autologous WT1-loaded DCs demonstrated WT1-specific CD8<sup>+</sup>, but not CD4<sup>+</sup> T-cell responses after vaccination, as shown by significant increases in WT1-specific interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ , but not IL-5 production. This vaccine-specific CD8<sup>+</sup> T-cell response was present in the long-term, but not in the non-long-term survivor group (Figure 4A-B-C). Notably, significant WT1-specific bifunctional TNF- $\alpha$ <sup>+</sup>/IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T-cell responses were also detected in the long-term survivor group (Figure 4D). In some long-term responders (UPN14) or survivors (UPN17, 34, 35), the proportion of IFN- $\gamma$ <sup>+</sup> and/or TNF- $\alpha$ <sup>+</sup> WT1-specific CD8<sup>+</sup> DILs was very high (range 5%-50%). IFN- $\gamma$ <sup>+</sup> and TNF- $\alpha$ <sup>+</sup> WT1-specific CD8<sup>+</sup>, but not CD4<sup>+</sup> DIL responses were significantly higher in patients vaccinated with the *DC-LAMP*-containing *WT1* constructs as compared to those vaccinated with the wild type *WT1* construct (data not shown).

## DISCUSSION

In this phase II study, we demonstrated clinical activity of autologous *WT1* mRNA-electroporated DC vaccination in patients with AML in remission and showed that this form of cancer vaccine therapy may offer overall survival (OS) benefit that is linked to the induction of *WT1*-specific CD8<sup>+</sup> T-cell immunity. The clinical response rate that was obtained in this study (43%) is of considerable interest, with 30% of molecular response (MR) and 13% of stable disease (SD). The high rate of patients achieving MR (9/30 patients) is an important finding, since these patients were otherwise destined to relapse based on their increased pre-vaccination *WT1* transcript levels.<sup>19,21-25</sup> In the SD response group (4/30 patients), *WT1*/DC vaccination was effective in temporarily halting AML progression (as characterized by stabilization of *WT1* transcript levels and stable blood values without blasts), thereby delaying the occurrence of florid relapse. SD is an unexpected response category in the context of AML, where relapses are normally characterized by exponentially increasing *WT1* transcript levels at a constant doubling time.<sup>19</sup> Similar observations of disease stabilization have been made in *WT1* peptide vaccine trials in AML,<sup>7,8,32</sup> indicating that SD should be included as a separate category in the response assessment of immunotherapeutic interventions in hematological malignancies as it is now routinely the case in the field of solid tumor immunotherapy.<sup>33</sup> Altogether, the data above indicate that *WT1*-targeted DC vaccination can be an effective strategy to prevent or delay relapse in AML, without the toxicity of allo-HSCT. This is an important finding in light of the growing number of elderly AML patients who are generally not considered candidates for allo-HSCT because of toxicity considerations.<sup>5</sup> Based on these results, a place for *WT1*/DC vaccination can also be foreseen for younger AML patients who do not proceed to allo-HSCT after standard chemotherapy because of refusal or lack of a suitable donor.

The OS data in this study compared favorably with current and new data from SEER and SALR, respectively. Importantly, this comparatively longer OS was observed not only in younger patients ( $\leq 65$  yr) but also in the bad prognosis older age category ( $> 65$  yr). Long-term OS were seen in both favorable and intermediate cytogenetic risk groups. OS and RFS also correlated with the clinical response to *WT1*/DCs. These observations further validate the use of *WT1* transcript levels as a suitable marker for leukemic residual disease and for monitoring the effect of therapy in AML. Increased OS in AML was recently found to correlate with reduced *WT1* mRNA levels and *WT1*-specific CD8<sup>+</sup> T-cell responses in a cohort of AML patients after chemotherapy and allo-HSCT.<sup>34</sup> The comparatively longer OS observed in AML patients vaccinated with *WT1*/DCs is consistent with a meta-analysis indicating that DC vaccine therapy can offer OS benefit in patients with solid malignancies, including melanoma, prostate cancer, glioblastoma multiforme and renal cell cancer.<sup>14</sup>

Intriguingly, 5-yr relative OS but not relapse-free survival (RFS) of the patients vaccinated with WT1/DCs compared favorably with the SALR data. Our data suggest that one major reason for the OS advantage is the unexpectedly high clinical response rate and long survival in the patients who had relapsed after WT1/DC vaccination. For AML patients in first relapse, the probability of achieving a second CR with salvage treatment (i.e. chemotherapy and/or allo-HSCT) has been reported to be 46%,<sup>35</sup> whereas the CR2 rate in our study was 73.3%. Likewise, the 5-yr OS rate of WT1/DC-vaccinated AML patients following first relapse was 36.8% in this study, which compares favorably to the 11% 5-yr OS rate of AML patients in first relapse that has been described in the literature.<sup>35</sup> These data suggest that WT1/DC vaccination can potentiate the response to subsequent treatment, providing an explanation for how vaccination contributes to prolongation of survival. A similar scenario has been reported for solid tumors, where improved clinical outcomes have been documented in patients who received chemotherapy after apparently failing immunotherapy as compared to patients who received chemotherapy alone.<sup>36-44</sup> This outcome may reflect synergism between immunotherapy and chemotherapy, the latter having not only anti-proliferative but also immunostimulatory effects.<sup>6,13</sup> The precise mechanisms underlying the anticancer synergy between tumor vaccines and chemotherapy are currently being investigated;<sup>41</sup> one potential mechanism involves the release of cytokines (such as TNF- $\alpha$ ) by vaccine-induced CD8<sup>+</sup> T-cells, which in turn enhances the ability of chemotherapy to induce apoptotic tumor cell death.<sup>45</sup>

In this study, clinical response and survival were found to be correlated with induction of WT1-reactive CD8<sup>+</sup> T-cell immunity by the DC vaccination, providing a mechanistic basis for the anti-leukemic activity of WT1/DCs. First, we found a correlation between long-term clinical response and increased circulating frequencies of poly-epitope WT1-specific tetramer<sup>+</sup> CD8<sup>+</sup> T-cells. The maintenance or increase of the frequencies of WT1-specific CD8<sup>+</sup> T-cells at relapse or progression, points towards antigen-driven immune activation associated with increasing exposure to the WT1 antigen at relapse<sup>46</sup> and with the continuation of the WT1/DC vaccination. This suggests that antigen-specific T-cell numbers may be necessary, but not sufficient to ultimately control AML. Second, we found a correlation between WT1-specific IFN- $\gamma$ <sup>+</sup> and/or TNF- $\alpha$ <sup>+</sup> DTH-infiltrating CD8<sup>+</sup> T-lymphocytes and long-term OS. This suggests that CD8<sup>+</sup> T-cell function is needed for long-term control of AML, at least in the immunotherapy setting. Since DTH was not performed in this study at the time of relapse, we cannot exclude that despite a maintenance or increase in WT1-specific cell numbers, their function may be deficient at that time.

In line with preclinical data,<sup>18</sup> 3 different *WT1* constructs were used in this study: a native full-length *WT1* construct, a *WT1* construct incorporating the lysosomal targeting signal of *DC-LAMP*, and a codon-optimized version of the latter construct. Theoretically, the

*DC-LAMP*-containing constructs would facilitate MHC class II antigen presentation and subsequent CD4<sup>+</sup> T-cell stimulation. Contrary to expectations, no statistical evidence for induction of WT1-specific CD4<sup>+</sup> T-cell immunity was found in the patients in whom the *DC-LAMP*-containing constructs were used. This is in contrast with a recently published study in melanoma, which used DCs loaded with melanoma antigen-encoding mRNA linked to a similar *DC-LAMP* construct.<sup>47</sup> The apparent lack of CD4<sup>+</sup> T-cell stimulation in our hands may be due to the fact that the strongly immunogenic WT1<sub>332-347</sub> MHC class II epitope<sup>48,49</sup> is not encoded by the *DC-LAMP*-containing constructs because they lack the *WT1* nuclear localization signal (NLS).<sup>18</sup> Nevertheless, even without a significantly increased stimulation of WT1-specific CD4<sup>+</sup> T-cells, the *DC-LAMP*-containing constructs did induce higher frequencies of WT1-specific CD8<sup>+</sup> T-cells as compared to the wild-type *WT1* construct, presumably as a consequence of higher cytoplasmic WT1 expression and MHC class I epitope presentation.<sup>18</sup> This, however, did not translate in superior clinical response rates, nor in improved survival outcome.

In summary, WT1-targeted DC vaccination can elicit anti-leukemia T-cell immunity in AML patients at very high risk of relapse. The induction of functional WT1-specific CD8<sup>+</sup> T-cells is a likely mechanism to help eliminate residual leukemic cells, decrease the likelihood of AML relapse and improve survival. Vaccination with WT1/DCs can therefore be considered as a non-toxic, post-remission strategy to prevent or delay relapse of AML in the adjuvant setting.

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## AUTHORSHIP AND CONFLICT-OF-INTEREST STATEMENTS

**Author contributions:** S.A., A.L.V.D.V., E.L.S., V.F.V.T., G.J., N.C., E.L., A.V.D., L.M., P.J., H.G., Y. Oka, F.F., Y. Oji, H.S., and Z.N.B. designed research. S.A., A.L.V.D.V., E.L.S., V.F.V.T., G.J., N.C., G.N., B.S., A.V.D., K.V., M.B.M., K.D., R.M., F.F., and Y. Oji performed research. G.J. contributed the data from the Swedish Acute Leukemia Registry. S.A., A.L.V.D.V., G.J., I.V., A.V., A.P.G., W.A.S., M.M.C., and Z.N.B. contributed and/or treated patients. K.V., M.B.M., E.G., M.L., H.G., D.A.P., K.L., F.F., Y. Oji, and H.S. contributed new and/or critical reagents and/or analytic tools. S.A., A.L.V.D.V., V.F.V.T., G.J., N.C., K.V., D.A.P., K.L., Y. Oka, Y. Oji, H.S., and Z.N.B. analyzed data. S.A., A.L.V.D.V., G.J., V.F.V.T., and Z.N.B. prepared the tables and/or figures. S.A., A.L.V.D.V., E.L.S., S.A., V.F.V.T., G.J., K.V., Y. Oji, and Z.N.B. wrote the final version of the report and all authors approved it.

**Conflict-of-interest disclosure:** V.F.V.T. and Z.N.B. are co-inventors of a patent covering the mRNA electroporation technique (WO/2003/000907 - Improved transfection of eukaryotic cells with linear polynucleotides by electroporation). Z.N.B. is a member of the Scientific Advisory Board of ExoCyte Therapeutics.

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TABLE 1




		<i>This study</i> <sup>◇</sup>			<i>SALR</i> <sup>‡</sup>
		All	Responders	Non-responders	
All ages		n=26	n=12	n=13	n=2495
OS	median	56.1 mo	n.r. [101.7 mo]	} <i>P</i> =0.0019 {	29.9 mo
	5-yr %	50.0 %	75.0 %		37.9 %
RFS	median	14.2 mo	59.6 mo	} <i>P</i> <0.0001 {	16.7 mo
	5-yr %	30.8 %	50.0 %		32.1 %
≤65 years		n=13	n=6	n=6	n=1542
OS	median	n.r. [101.7 mo]	n.r. [115.9 mo]	} <i>P</i> =0.0179 {	74.8 mo
	5-yr %	69.2 %	100 %		51.7 %
RFS	median	58.0 mo	n.r. [114.8 mo]	} <i>P</i> =0.0059 {	30.3 mo
	5-yr %	46.2 %	66.7 %		43.9 %
>65 years		n=13	n=6	n=7	n=953
OS	median	32.2 mo	59.2 mo	} <i>P</i> =0.0270 {	18.2 mo
	5-yr %	30.8 %	50.0 %		18.0 %
RFS	median	11.5 mo	41.0 mo	} <i>P</i> =0.0043 {	10.6 mo
	5-yr %	15.4 %	33.3 %		14.9 %

<sup>◇</sup>, all newly diagnosed acute myeloid leukemia (AML) patients in first complete remission (CR1) enrolled in the dendritic cell (DC) vaccination studies NCT00834002 and NCT00965224; three AML patients who were in second or third CR (UPN02, UPN16 and UPN34) and one AML patient who did not reach CR1 (UPN20) were excluded from analysis. <sup>‡</sup>, all study patients who responded to DC vaccination; patient UPN22 had an undefinable response and was excluded from analysis. <sup>§</sup>, observed survival of newly diagnosed AML patients in CR1 enrolled in the Swedish Acute Leukemia Registry (SALR), diagnosis from 1997-2014. OS, overall survival calculated from time of diagnosis. RFS, relapse-free survival calculated from start of CR1. Median, median survival expressed in months (mo). 5-yr %, 5-year survival percentage (%). n.r., median survival not reached. [ number ], median follow-up time in months (mo). { *P*= }, *p* value (Gehan-Breslow-Wilcoxon test) of the survival comparison between "Responders" and "Non-responders".

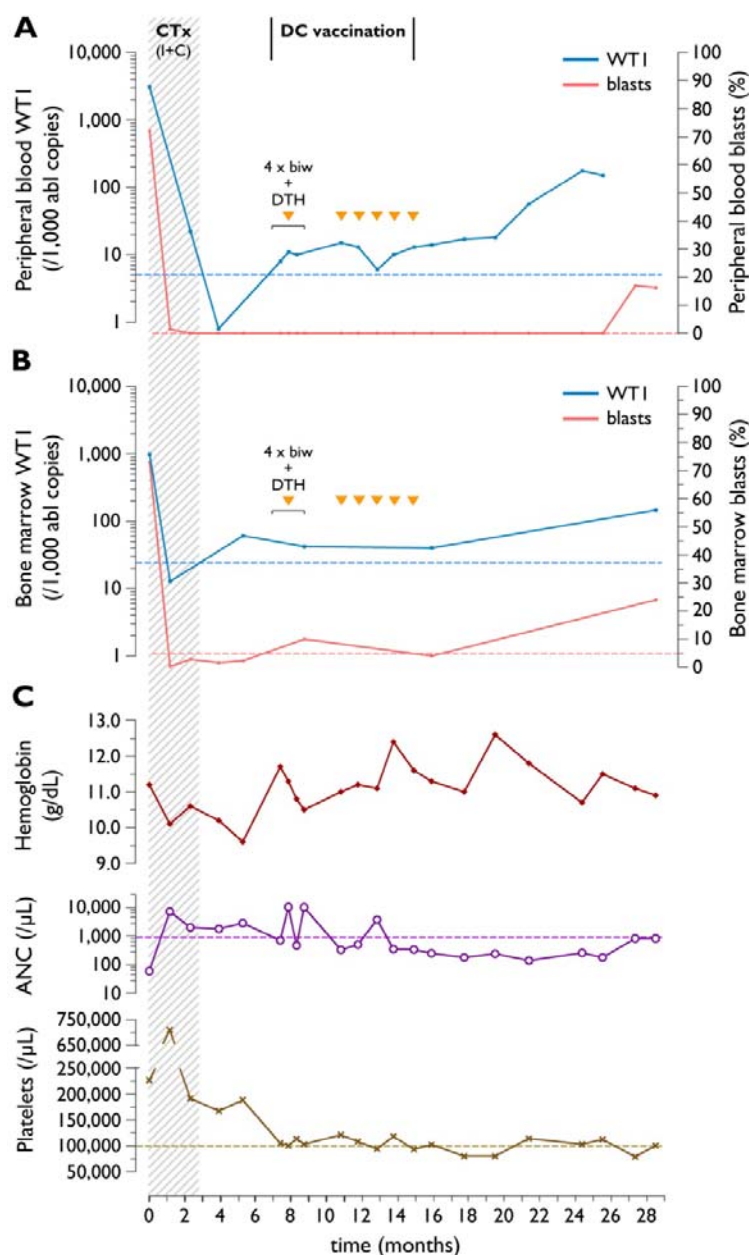
**Table 1. Overall survival (OS) and relapse-free survival (RFS) of AML patients in first complete remission (CR1) enrolled in this study as compared to the Swedish Acute Leukemia Registry (SALR).**

## FIGURES AND FIGURE LEGENDS

**Figure 1. The three different *WT1* constructs used to generate mRNA for electroporation into DCs and their corresponding clinical responses and survival outcome.** Construct 1 (“WT1”) encodes full-length *WT1* (A), construct 2 (“WT1-DC-LAMP”) includes a Sig-DC-LAMP major histocompatibility complex (MHC) class II-skewing signal with deletion of the *WT1* nuclear localization signal (B) and construct 3 (“WT1-DC-LAMP-OPT”) is a codon-optimized version of construct 2 (C). For further details, see reference<sup>18</sup> MR, molecular remission; SD, stable disease; Undef., undefinable; median OS, median overall survival calculated from the start of *WT1*/DC vaccination; the values between square brackets represent median follow-up; mo, months; 5-yr % OS, 5-year overall survival percentage from the start of *WT1*/DC vaccination; UTR, untranslated region; NLS, nuclear localization signal. The full color blue bars represent the remaining coding sequence of *WT1* in constructs 2 and 3. The SD phase in UPN35 started during the administration of DCs electroporated with construct 3 (see Table S1).

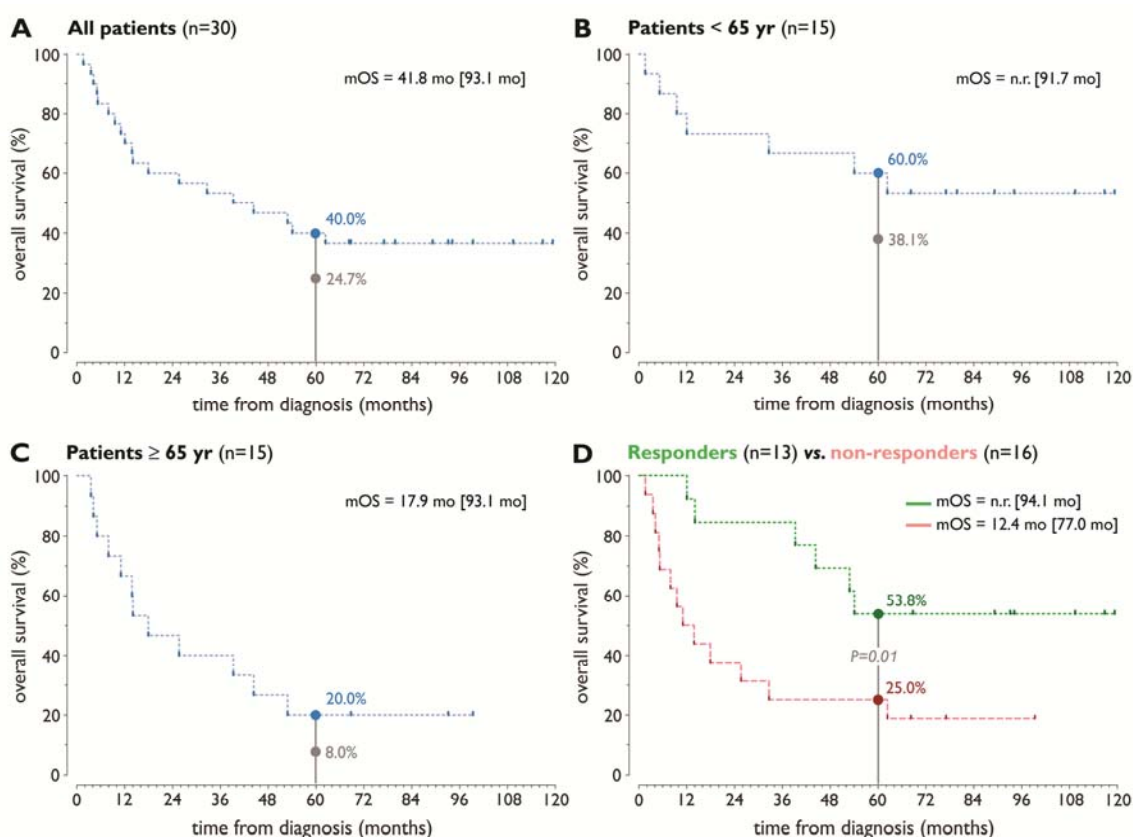
	CLINICAL RESPONSE				SURVIVAL	
	Response MR	SD	No response	Undef. response	median OS	5-yr % OS
<b>A CONSTRUCT 1: “WT1 group”</b> 	7	1	8	0	23.3 mo [109.4 mo]	31.3 %
<b>B CONSTRUCT 2: “WT1-DC-LAMP group”</b> 	2	1	2	1	69.2 mo [89.2 mo]	50.0 %
<b>C CONSTRUCT 3: “WT1-DC-LAMP-OPT group”</b> 	0	2	6	0	50.8 mo [68.7 mo]	50.0 %
	n=13		n=16	n=1		

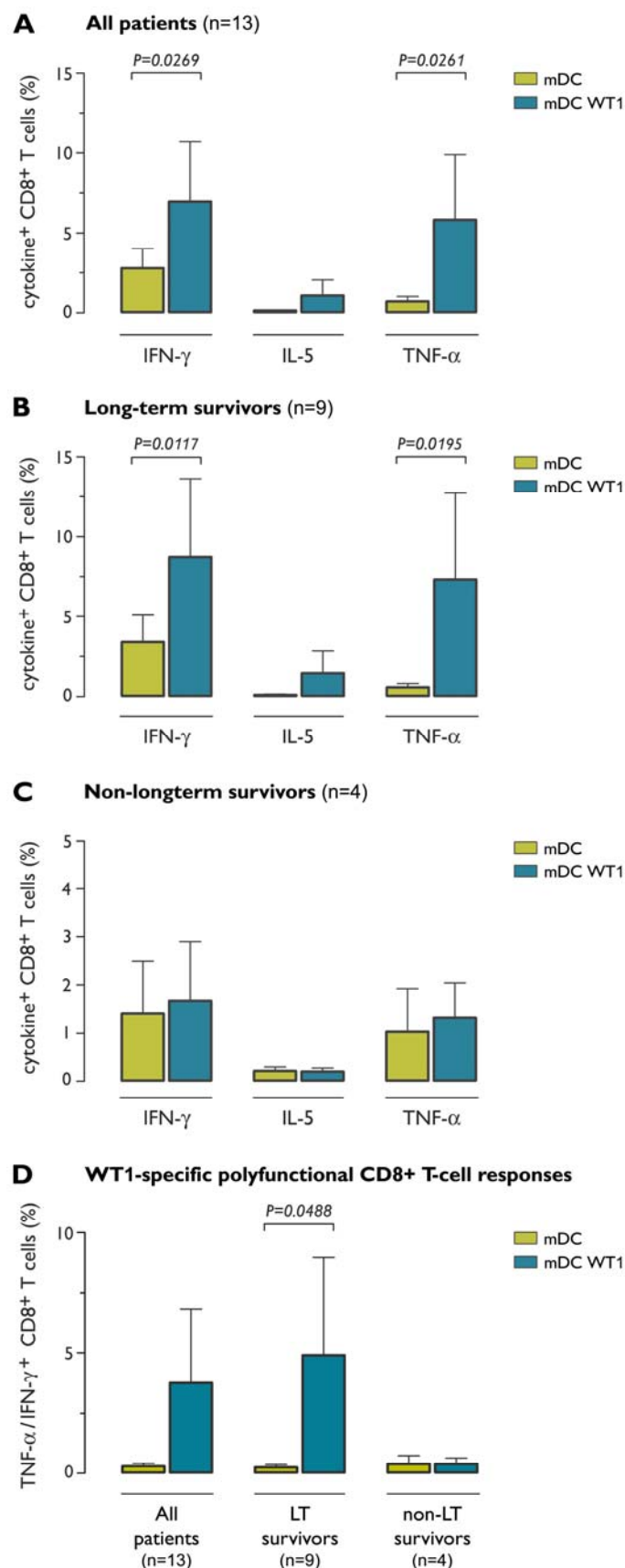
**Figure 2. Stable disease (SD) in patient UPN33 during (arrows) and after WT1/DC vaccination.** *WT1* transcript levels (determined by the Ipsogen *WT1* ProfileQuant Kit) in blood (A) and bone marrow (B) were above background (indicated by the dotted blue line) but remained stable, and the bone marrow blast count normalized (normal value indicated by the dotted pink line). Blood values (C) showed pancytopenia at the start of DC vaccination, but a normal hemoglobin level (without transfusions) at the end of the SD period (at 19 months); neutropenia was treated with granulocyte colony-stimulating factor (G-CSF). CTx (I+C), polychemotherapy (induction + 2 consolidations); 4 x biw + DTH, period of the first 4 biweekly WT1/DC vaccinations and DTH; ANC, absolute neutrophil count.





**Figure 3. Kaplan-Meier curves of the overall survival data.** The values on the curves are 5-yr relative survival from the start of WT1/DC vaccination; the values underneath in gray (A, B, C) are 5-yr relative survival data from SEER (observed survival of newly diagnosed AML patients included in Surveillance, Epidemiology and End Results (SEER)\*Stat Database “Incidence - SEER 18 Regs Research Data + Hurricane Katrina Impacted Louisiana Cases, Nov 2014 Sub (1973-2012 varying)”, whereby the following case selection criteria were applied: age (min. age 30 years, max. age 79 years), race (white) and year of diagnosis (2005-2012); the patient with an undefinable response (UPN22) was not included in figure 3D. mOS, median overall survival; the values between square brackets represent median follow-up; mo, months; n.r., not reached.





**Figure 4. Intracellular cytokine staining of CD8<sup>+</sup> DILs after restimulation with mature DCs alone ('mDC') or WT1 mRNA-electroporated DCs ('mDC WT1').** The WT1-specific T-cell cytokine response was evaluated by comparing 'mDC WT1' with 'mDC' (all patients examined: UPN03, 05, 06, 08, 14, 17, 21, 22, 29, 30, 34, 35, 47; long-term survivors: UPN06, 08, 14, 17, 21, 22, 29, 34, 35; non-long-term survivors: UPN03, 05, 30, 47). IFN- $\gamma$ <sup>+</sup>, IL-5<sup>+</sup> or TNF- $\alpha$ <sup>+</sup> CD8<sup>+</sup> T-cells are shown for all patients (A), long-term survivors (B) and non-long-term-survivors (C). Polyfunctional TNF- $\alpha$ <sup>+</sup>/IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> T-cells are shown in the same patients (D). \*, statistically significant difference; LT, long-term.

Figure 1


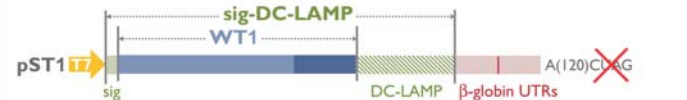
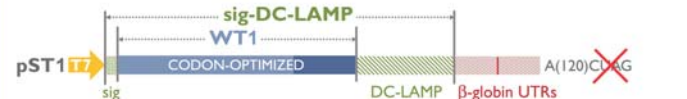
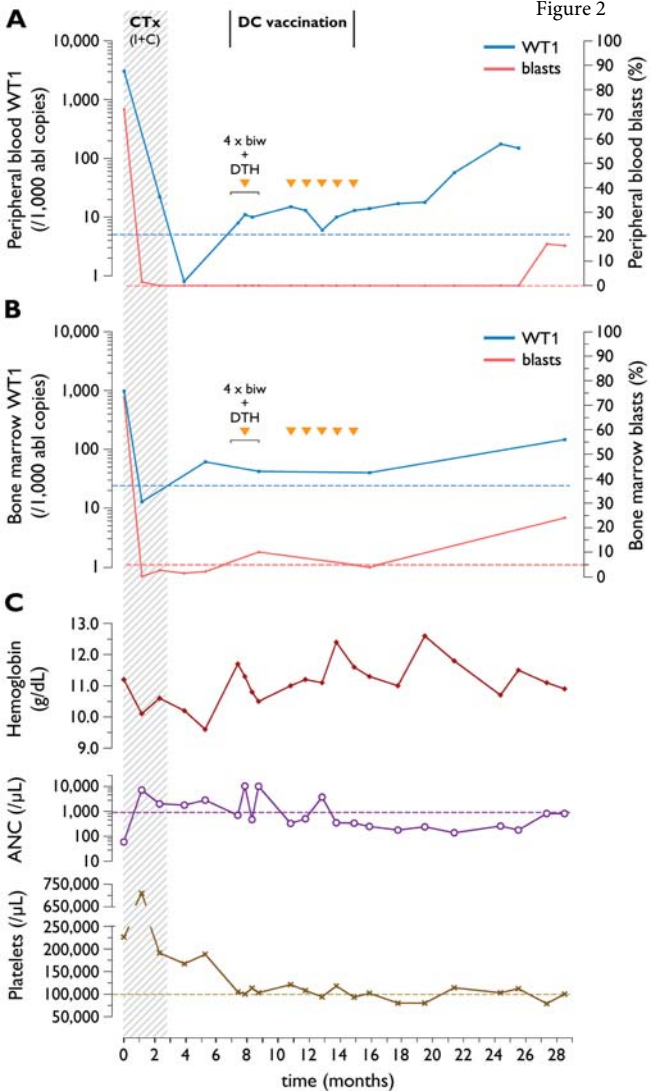
	CLINICAL RESPONSE				SURVIVAL	
	Response MR	SD	No response	Undef. response	median OS	5-yr % OS
<b>A CONSTRUCT 1: "WT1 group"</b> 	7	1	8	0	23.3 mo [109.4 mo]	31.3 %
<b>B CONSTRUCT 2: "WT1-DC-LAMP group"</b> 	2	1	2	1	69.2 mo [89.2 mo]	50.0 %
<b>C CONSTRUCT 3: "WT1-DC-LAMP-OPT group"</b> 	0	2	6	0	50.8 mo [68.7 mo]	50.0 %
	n=13		n=16	n=1		

Figure 2



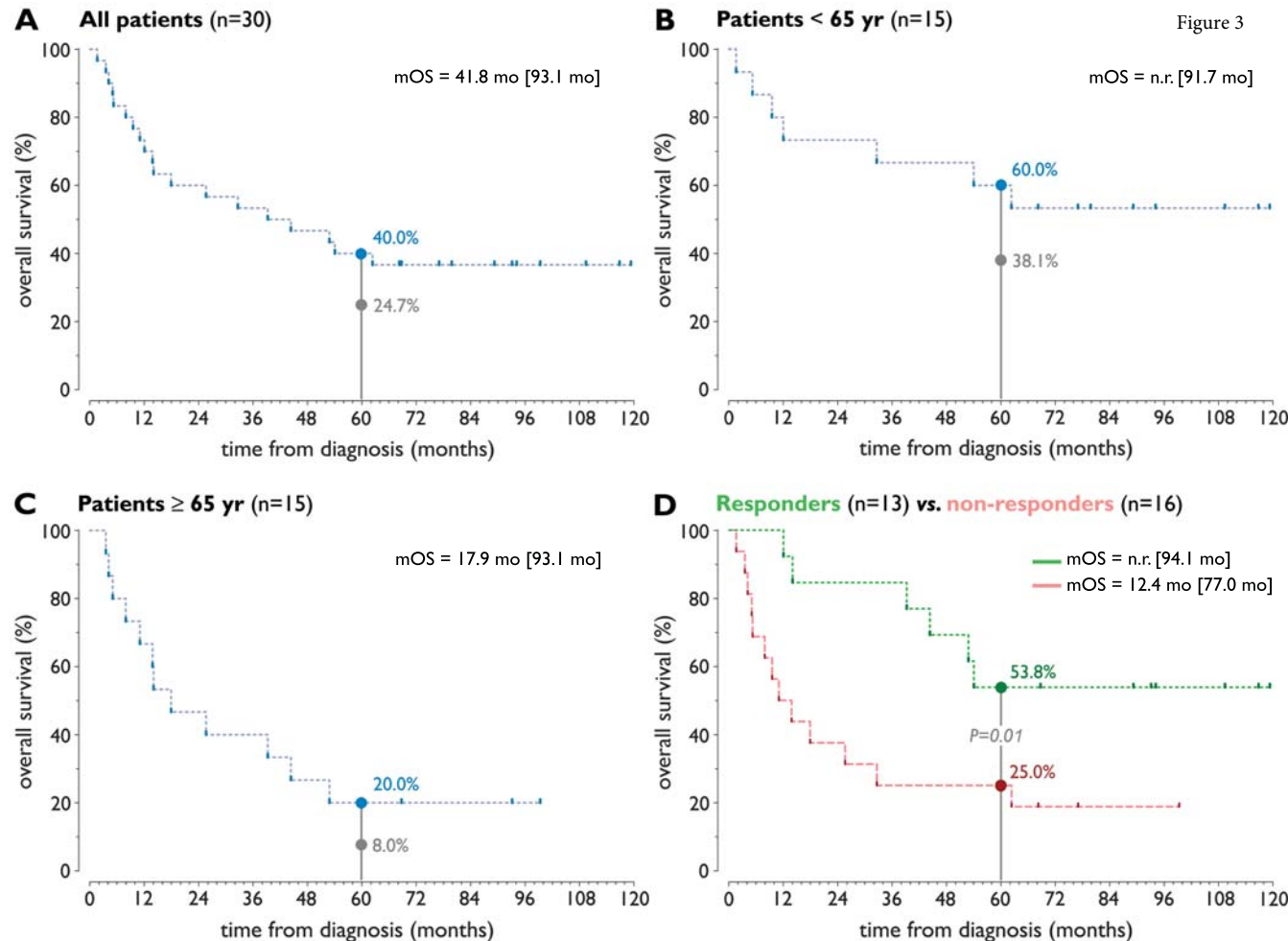


Figure 4

